PATENT
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Applicant:	Xinnian Dong et al.	Art Unit:	1638
Serial No.:	08/908,884	Examiner:	A. Nelson
Filed:	August 8, 1997	Customer No.:	21559
Title:	ACQUIRED RESISTANCE GENES AND USES THEREOF		

Assistant Commissioner For Patents
Washington, D.C. 20231

DECLARATION OF DR. XINNIAN DONG

1. I am a co-inventor of the pending claims in the above-referenced application.

2. I have read the Office Action mailed on March 12, 2001.

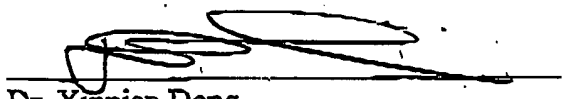
3. Prior to May 8, 1996, I, along with my co-inventors, determined that the *NPR1* gene resided on a yeast artificial chromosome ("YAC") clone designated "yUP19H6." In addition, prior to that date, we determined that three RFLP markers--m305, yUP21A4L, and g8020--were closely linked to the *NPR1* gene. The experiments we carried out to accomplish the map-based positional cloning of the *NPR1* gene on the yUP19H6 YAC clone are as described in our patent application at pages 33 (line 1) – 35 (line 16) (Exhibit 1). A schematic illustration showing the position of the *NPR1* gene on the yUP19H6 YAC clone relative to the genetic markers g8020, m305, and 21A4L (designated "yUP21A4L") is shown in Figure 1 (Exhibit 2) of the application. To evidence our possession of the yUP19H6 YAC clone prior to May 8, 1996, I attach a copy of a laboratory notebook page (Exhibit 3) outlining an experiment designed to

identify subclones of the yUP19H6 YAC clone that contained the *NPR1* gene. As described on this notebook page, colony lifts of a yUP1946 library were prepared and probed with the m305 and 21A4L markers. In addition, the notebook entry indicates that the colony lifts were also to be probed with the g8020 marker. Because the purpose of this experiment was to subclone the *NPR1* gene from the larger YAC clone, it evidences our possession of a piece of DNA that includes the *NPR1* gene.

4. The above experiments were carried out in the United States prior to May 8, 1996. Any date appearing on this notebook page has been redacted, but is prior to May 8, 1996.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patents issued thereon.

Date: 05/10/02



Dr. Xinnian Dong

EXHIBIT 1
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Map-Based Positional Cloning of the *Arabidopsis NPR1* Gene

To map the *NPR1* gene, a genetic cross was made between the *npr1-1* mutant (present in the Columbia ecotype (Col-0) which carried the *BGL2-GUS* reporter gene) and the wild-type (present in Landsberg *erecta* ecotype (La-*er*) which carried the *BGL2-GUS* reporter gene). F3 families from this cross that are homozygous for this mutation at the *NPR1* locus were identified by their lack of expression of *BGL2-GUS* when grown on plates containing 0.1 mM INA. Expression of the GUS reporter gene was detected by a chromographic assay of GUS activity using the substrate 5-bromo-4-chloro-3-indolyl glucuronide according to standard techniques (Cao et al., *Plant Cell* 6:1583-1592, 1994 and Jefferson *Plant Mol. Biol. Reporter* 5:387-405, 1987). The leaf tissues of these F3 *npr1-1* progeny pools (from thirty to forty two-week-old seedlings) were collected and frozen in liquid nitrogen. From the frozen tissues, genomic DNA preparations were made as described by Dellaporta et al. (*Plant Mol. Biol. Reporter* 1:19-21, 1983) and used to determine the genotypes of various restriction fragment length polymorphism (RFLP) and codominant amplified polymorphic sequence (CAPS) (Konieczny and Ausubel, *Plant J.* 4:403-410, 1993) markers. The frequencies of recombination between the *NPR1* locus and the RFLP and CAPS markers were used to determine the position of the *NPR1* gene according to conventional methods.

As shown in Fig. 1, the *NPR1* gene was mapped to *Arabidopsis* chromosome 1, and found to reside between the CAPS marker GAP-B (~22.70 cM on the centromeric side of the *NPR1* gene) and the RFLP marker m315 (~7.58 cM on the telomeric side of the *NPR1* gene).

To carry out fine mapping of the *NPR1* gene, new CAPS and RFLP markers were generated from clones that the genetic maps in the AtDB database (http://genome-www.stanford.edu/Arabidopsis/) showed were located between *GAP-B* and *m315*. Cosmid *g4026* (CD2-28, *Arabidopsis* Biological Resource Center, The Ohio State University, Columbus, OH) was cut with the restriction enzyme *Eco*RI and a 4-kb

fragment was used to identify a polymorphism between Col-0 and La-*er* after the genomic DNA was digested with *Hind*III. Using this RFLP marker, six heterozygotes were detected among the twenty-three F3 families that were heterozygous at *GAP-B*. None were found among the seven F3 families that were heterozygous at *m315*. Therefore, g4026 is ~5.92 cm on the centromeric side of the *NPR1* gene. Cosmid *g11447* (obtained from the collection of Dr. Howard Goodman at the Massachusetts General Hospital (Nam et al., *Plant Cell* 1:699-705, 1989)) was used to generate a CAPS marker. End-sequences of an 0.8-kb *Eco*RI fragment were used to design PCR primers (primer 1: 5' GTGACAGACTTGCTCCTACTG 3' (SEQ ID NO:15); primer 2: 5' CAGTGTGTATCAAAGCACCA 3' (SEQ ID NO:16) which amplified a fragment displaying a polymorphism when digested with the *Eco*RV restriction enzyme. Among the 436 *npr1-1* F3 progeny tested using this newly generated CAPS marker, seventeen heterozygotes were discovered. Since these heterozygotes were all homozygous Col-0 for the *GAP-B* locus, the *g11447* marker was placed ~1.95 cM on the telomeric side of the *NPR1* gene.

There are a number of RFLP markers mapped between *g11447* and *g4026*. The first marker tested was *m305* (designated CD1-11, *Arabidopsis* Biological Resource Center, the Ohio State University, Columbus, OH (Chang et al., *Proc. Natl. Acad. Sci., USA* 85:6856-6860, 1988)). A 5-kb *Eco*RI fragment isolated from the *m305* lambda clone was further subcloned using *Sall/Xba*I and the end-sequences of a 1.6-kb fragment were used to design PCR primers (primer 1: 5' TTCTCCAGACCACATGATTAT 3'(SEQ ID NO:17); primer 2: 5' TGAAGCTAATATGCACAGGAG 3' (SEQ ID NO:18)). The resulting PCR fragment amplified using these primers was digested with *Hae*III to detect a polymorphism. Among the 305 *npr1-1* progeny examined using this *m305* CAPS marker, no heterozygotes were found, indicating that the *m305* marker lies extremely close to *NPR1*.

A partial physical map of chromosome I (http://cbil.humgen.upenn.edu/~atgc/ATGCUP.html) showed a YAC contig that includes *m305*. The YACs in this contig, as well as left-end-fragments of YAC clones yUP19H6, yUP21A4, and yUP11H9 were obtained from Dr. Joseph Ecker at the University of Pennsylvania. The yUP19H6L end-probe was found to detect an *Rsa*I polymorphism, and five recombinants were identified among the *GAP-B* recombinants on the centromeric side of the *NPR1* gene (as shown by the vertical arrows in Fig. 1). The yUP11H9L end-probe was found to detect a *Hind*III polymorphism, and one heterozygote was found among the seventeen recombinants for *g11447* on the telomeric side of the *NPR1* gene (as shown by a vertical arrow in Fig. 1). Since yUP11H9L hybridized with the yUP19H6 YAC clone, these results showed that the *NPR1* gene is located on yUP19H6. In addition to *m305*, yUP21A4L (detects an *Eco*RI polymorphism) and *g8020* (a 1.3-kb *Eco*RI fragment that detects a *Hind*III polymorphism) were found to be very closely linked to the *NPR1* gene with no recombinants identified. *m305*, yUP21A4L, and *g8020* all hybridized to the yUP19H6 YAC clone, further supporting the conclusion that yUP19H6 contains the *NPR1* gene.

Construction of a Cosmid Library from the YAC Clone yUP19H6

A genomic DNA preparation was made from the yeast strain containing the YAC clone yUP19H6. This DNA was partially digested with the restriction enzyme *Taq*I, size selected on a 10-40% sucrose gradient, and cloned into the *Cla*I site of the binary vector, pCLD04541 (obtained from Dr. Jonathan Jones (Bent et al., *Science* 265:1856-1860, 1994)). The pCLD04541 vector is a standard transformation vector used for preparing cosmid libraries. This plasmid carries a T-DNA polylinker region, and tetracycline and kanamycin resistance markers.

The cosmid clones were packaged into bacteriophage lambda particles using a commercial packaging extract (Gigapack XL, Stratagene, LaJolla, CA) and introduced into *E. coli* strain DH5α according to the instructions of the supplier. The resulting

EXHIBIT 2
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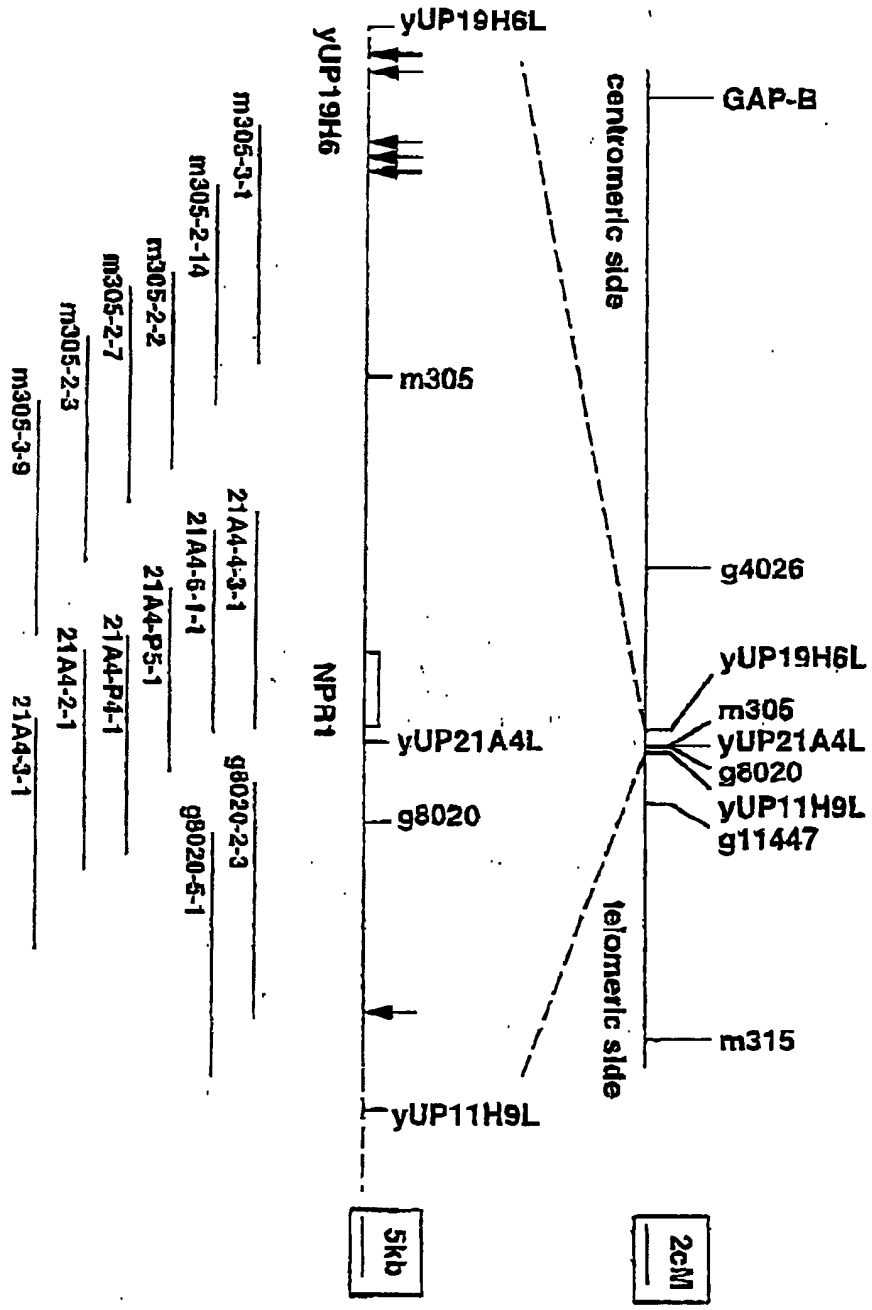

FIG. 1

EXHIBIT 3
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- colony lifts the 10 137mm plates of Yap 4946 library from Jane
- prepare the solutions for polyA+ mRNA extraction
- Digest 6 hets w/ HincII (to be probed w/ gf8020 1.3kb)

- ~~Probe~~ Probe 43 82mm discs w/ 1430J
  10 137mm discs w/ 21A4L
- Run 1.0% gel for 6 hets — Southern
- sterilize seeds of 6 hets
- grind tissues (RNA)
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